DESCRIPTION

CULTURING SYSTEM, DETECTING METHOD, AND DETECTION/ANALYSIS SYSTEM AND DETECTION METHOD FOR CANCER CELL COLONIES

TECHNICAL FIELD

The present invention relates to a detection/analysis system for cancer cell colonies, a detection method for cancer cell colonies, and a culturing system using the same. More particularly, the invention relates to a culturing system according to a soft agar double layer method composed of a soft agar, and a system and a method which semi-automatically conduct cell cultivation using the same and analyzes the cultured cell to detect the cancer cell colonies.

BACKGROUND ARTS

In spite of remarkable advance of medical techniques, cancer is the top of the causes of the person's death in Japan since 1981. Consequently, societies has being interested in preventing cancer,. In this connect, first of all, the development history of cancer should be known for finding a way to prevent cancer.

In general, carcinogenic substances are taken into a body, almost all of them are metabolized in the body and they are exhausted out of the body. The remaining parts of the carcinogenic substances in the body finally cause diseases, cancer, via three stages. First, these carcinogenic

substances damage cell genes within a short period of time (for example, a few days) to cause mutant cells. The mutant cells become precancerous cells after a latent period of from several years to several ten years. Finally, the precancerous cells are rapidly growing (for example, one year) to be cancer. As described above, since we do not feel anything and it is difficult for present medical technique to detect a cancer until cells are converted into the precancerous cells, our remaining days are short upon being diagnosed to be cancer.

On the other hand, the consideration has been attracted that major parts of causes for cancer are our environment, particularly eating habits. For long life and heath, the relation between foods and prevention of cancer has being strongly interested.

However, since long periods are required for converting mutant cells into cancer cells, in order to prevent cancer, the conversion stages must be continuously inhibited over a prolong period of time. At the present, it is very difficult to effectively verify the prevention of cancer through food, counting on epidemiological verification. Consequently, development of an effective verification method for preventing caner through food materials and investigation for its functional mechanisms are considered to be increasingly required.

As one approach to develop an effective verification method for preventing caner through food materials and to

investigate functional mechanisms, Proceeding of the National Academy of Science of the United States of America Vol. 98, No. 13, pp 7510-7515 discloses that cancerous change of normal cells through a carcinogenic inducer agent and proceeding of cancer cells verified by forming colonies of cancer cells on an agar medium, and counting number of colonies.

However, the prior art is disadvantageous in concentration and thickness of the agar medium making it difficult to obtain the number of cell colonies in a stable manner. Also, the culturing period is very long, which requires from 20 to 30 days. Also, the prior art microscopically observes the colonies by human's eyes. This can count the number of colonies, but makes it impossible to analyze the size of each colony and distribution of colonies. Also, much more time and labors are required for counting, there are a lot of individual differences of the results of counting depending upon the examiners, leading to a problem in terms of accuracy. The problems remain in this verification method. In addition to count colonies with this verification method, there is also a problems in terms of requirement of skill of the examiner.

Consequently, an object of the present invention is to provide a system for detecting and analyzing cancer cell colonies, which can examine carcinogenic or anti-carcinogenic functions of cell carcinogenic inducers, which are a cause for cancer, in an environment, chemicals and foods for suppressing cancerous change of normal cells in a rapid and accuracy manner.

SUMMARY OF THE INVENTION

We have serious studied and investigated in light of above situations, have discovered that when culture conditions of cells, which will cancerously change normal cells, are standardized, when cells, which have cancerously changed according the standard, are taken into a calculating means, and when the taken data is specifically operated, the object described above can be solved, resulting in the present invention.

According to the first aspect there is provided a culturing system for caner cell colonies composed of a bottom layer having a thickness of 2.4 mm comprising a medium, a soft agar having a concentration of from 0.5% to 0.6%, and at least one substance selected from the group consisting of carcinogenic inducers and anti-carcinogenic agents; and a top layer having a thickness of 1.6 mm comprising a medium, a soft agar having a concentration of from 0.3% to 0.4%, and cells, the culturing system being prepared according to agar double layered culturing method.

In the culturing system according to the first aspect, it is important that the bottom layer and the top layer have prescribed configurations, respectively. Specifically, by making the culturing system specitic conditions, the results of detection and analysis can be obtained in a reproductive manner.

In the first aspect, the cells contained in the top layer

are preferably mouse neonatal skin cell JD6 line.

JB6 is a cell line established from a primary culture. This cell is non-neoplastic and has an ability for forming soft agar colonies only with a treatment of a carcinogenic promoter (TPA, TNF-alpha, EGF). This cell gains colony formation ability only with one treatment with TPA, is promoted and is in precancerous conditions; thus, it is considered to be a material superior to the detection system for the cancerous promotion and the analysis of the functional mechanism thereof. Also, many of compounds, which exhibited suppression of cancerous change in the detection system according to the present invention have been demonstrated to have similar effect for suppressing cancerous change at a test on animal. Consequently, the detection system is a system, which is attractive as means for rapidly detecting an effect for suppressing cancerous promotion.

According to the second aspect, there is provided a detection/analysis system for detecting cancer cell colonies utilizing a culturing system for caner cell colonies composed of a bottom layer having a thickness of 2.4 mm comprising a medium, a soft agar having a concentration of from 0.5% to 0.6%, and at least one substance selected from the group consisting of carcinogenic derivative substances and anti-carcinogenic agents; and a top layer having a thickness of 1.6 mm comprising a medium, a soft agar having a concentration of from 0.3% to 0.4%, and cells, the culturing system being prepared according

to agar double layered culturing method, said detection/analysis system comprising an optical microscope for observing the cancer cell colonies cultured by the culturing system; electric-data conversion means for converting an image from the optical microscope into an electric data; and a computer system for processing the electric data converted by the electric-data conversion means; wherein said computer system stores a program which converts the electric data into a gray scale, makes a calibration, and subtraction, converts the data into binary data through a single threshold value, to thereby analyze it for at least one item selected from the group consisting of presence or absence of colony(ies), number of colonies; and distribution of colonies.

This configuration makes it possible to rapidly and surely detect cell carcinogenic agents in an environment which will induce cancerous change of cells or suppress chemicals and foods which will suppress cancerous change of cells.

In the second aspect, the computer system preferably possesses the results of analysis of standard data obtained from any of known carcinogenic inducers, so that the results obtained in at least one substance selected from the group consisting of carcinogenic inducers and anti-carcinogenic substances are compared with the results of analysis of standard data obtained from any of known carcinogenic inducers.

In such a configuration, taking any of known components as a standard, behaviors of cancerous change of unknown

component can easily be examined. The term " behaviors of cancerous change" used herein means that the substance has either of a function for suppressing cancerous change and a function for inducing cancerous change.

In the second aspect, the known substance is selected from the group consisting of TPA, TNF-alpha, and reactive oxygen species.

The behaviors of these substances have been known, comparison with any of these substances as a standard makes it easy to understand the behavior of unknown substance whether it is carcinogenic or anti-carcinogenic.

According to the third aspect, there is provided a detection/analysis method for cancer cell colonies utilizing the detection and analysis system according to the present invention comprising the following stages: (A) selecting a substance having a carcinogenic function or an anti-carcinogenic function to prepare the culturing system; (B) culturing cancer cell colonies within said culturing system under prescribed conditions for a prescribed time; (C) sending a data of the cancer cell colonies cultured via the microscope and the electric data conversion means to the computer system as an electric data; (D) making the sent electric data gray scale, calibration, and subtraction; and converting it into a binary data through a single threshold value, and analyzing it for at least one item selected from the group consisting of presence or absence of colony(ies), number of colonies; and distribution

of colonies.

This configuration makes it possible to rapidly and surely detect cell carcinogenic agents in an environment which will induce cancerous change of cells or suppress chemicals and foods which will suppress cancerous change of cells.

In the stage (A) of the third aspect, the substance having a carcinogenic function or an anti carcinogenic function is preferably a food or a substance originated from a food.

The carcinogenic function or anti-carcinogenic function possessed by food is one of the most interested matters. The present invention can analyze the behaviors of cancerous change of a food or a substance originated from a food.

In the stage (B) of the third aspect, the culturing conditions of the culturing system are preferably at a temperature of about 37 degree C, under 5% carbon dioxide gas atmosphere over a period of from 15 to 30 days.

The standardization of the culturing conditions leads to analysis with good accuracy under the same conditions and with good reproductivity.

Furthermore, in the stage (D) of the third aspect, it is preferable that the analysis is executed by using an image analyzing software executed on this computer system to make a transparency of the agar gel uniform, to treat scattered light of the microscope to analyze at least one item selected from the group consisting of shapes, sizes, and number of the colonies, and size distribution thereof, that an image

differential treatment is carried out to distinguish the colonies from dust, that the spot having a long diameter/short diameter ratio not more than 1.6 is judged to be the cancer cell colony.

The use of such an image analyzing software can make the analysis with good accuracy and with good reproductivity. Also, the use of such an image analyzing software can carry out the analysis without high skill.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic view outlining the detection and analysis system for cancer cell colonies according to the embodiment;

FIG. 2 is a graph showing the results of measuring concentrated liquid of sweet potato juice added to the bottom layer of culturing system utilizing the detection and analysis system for cancer cell colonies according to the embodiment;

FIG. 3 is a graph showing the results of measuring blueberry anthocyanin added to the bottom layer of culturing system utilizing the detection and analysis system for cancer cell colonies according to the embodiment;

FIG. 4 is a graph showing the results of measuring blueberry Rain originated from Chinese medicine Rheum officinale (Chinese rhubarb) " added to the bottom layer of culturing system utilizing the detection and analysis system for cancer cell colonies according to the embodiment;

FIG. 5 is a graph showing the results of measuring peonidin malvidin, pelargonoidin, cyaniding and delphinidin contained in anthocyanidin respectively added to the bottom layer of culturing system utilizing the detection and analysis system for cancer cell colonies according to the embodiment;

BEST MODES FOR CARRYING OUT THE INVENTION

Embodiments of the present invention will now be described.

First, the outline of the detection/analysis system for caner cell colonies according to the present invention is shown in FIG. 1.

Referring to FIG. 1, the detection/analysis system for caner cell colonies according to the present invention is mainly composed of a culturing system 1 based on a prescribed agar double layer method, and a detection system 2 for detecting and analyzing the cancerously changed cells.

(Culturing System)

The culturing system 1 used in the present invention comprises a bottom layer 1 having a prescribed size comprising a medium, a soft agar and at least one substance selected from the group consisting of carcinogenic inducers and anti-carcinogenic agents; and a top layer 12 having a prescribed size comprising a medium, a soft agar, and cells, and the culturing system 1 is prepared according to agar double layered culturing method.

The medium used in the bottom layer 11, which can be used, may be suitably selected from among the conventionally known media, and an example includes EMEM (Eagle's minimum essential medium) plus 5% fetal bovine serum. Although the conventionally known soft agar medium can be used as the soft agar medium in the bottom layer 11, those from the same maker can be preferably used in terms of reproductivity. In the present invention, the concentration of the soft agar making up the bottom layer 11 is set at a constant value, for example, from 0.5 to 0.6% and preferably from 0.5%. The thickness is also set at a constant, for example, 2.4 mm. A prescribed amount of a carcinogenic inducer or an anti-carcinogenic agent is added to the bottom layer 11 thus prepared to prepare the bottom layer 11.

The concentrations and the thickness of the agar gels of the upper layer 13 and the bottom layer 12 should be somewhat transparent for the purpose of microscopically taking an image. For this reason, the concentrations and the thickness of the agar gels are examined to select concentrations and the thickness of the agar gels having no influence upon cell cultivation and capability of microscopically taking an image.

The carcinogenic inducers, which can be used herein are known cancerous inducers whose effect for cancerously changing cells is well established such as TPA, TNF-alpha, and reactive oxygen species, and cancerous inducers whose effect for cancerously changing cells is not known. As described later

on, the culturing system 1 having a known cancerous inducer added thereto prepared under prescribed conditions is used to conduct cultivation whereby the data detected and analyzed is obtained, which is used as a standard data. The same amount of an unknown component, which is to be detected and analyzed, is added to the culturing system 1, and the resulting data is compared with the standard data, making it possible to analyze the cancerous effect of the unknown component.

Similarly, any of anti-cancerous materials such as various medical anti-cancerous agents and anti-cancerous foods may be added to the bottom layer 11. Also, both an cancerous inducer and an anti-cancerous material may be added to the bottom layer 11 to analyze the anti-cancerous effect.

The upper layer 12 in the culturing system 1 of the present invention is composed of a soft agar having a prescribed size and a prescribed concentration and cells for which cancer cells grow.

The soft agar making up the upper layer 12 is required to set the concentration, for example, at from 0.3 to 0.4%, and to set the thickness, for example, at 1.6 mm.

The cells used herein are not specifically restricted, but is preferably JB6 line.

JB6 is a cell line established from a primary culture (N.H. Colburn et al., Nature, 281, 589 (1979)). This cell is non-neoplastic and has an ability for forming soft agar colonies only with a treatment of a carcinogenic promoter (TPA, TNF-alpha,

EGF). This cell gains colony formation ability only with one treatment with TPA, is promoted and is in precancerous conditions; thus, it is considered to be a material superior to the detection system for the cancerous promotion and the analysis of the functional mechanism thereof (see Z. Dong., et al., Proc. Natl. Acad. Sci. USA, 91, 609 (1994); Z. Dong et al., Mol Carcinog., 19, 204 (1997); C. Haang et., al. Proc. Natl. Acad Sci. USA, 95, 156 (1998); and J.Y. Chung et al., Cancer Res., 59, 4610 (1999)). Also, many of compounds, which exhibited suppression of cancerous change in the detection system similar to the detection system according to the present invention, which will be described later on, have been demonstrated to have similar effect for suppressing cancerous change at a test on animal .(M.R. Young, Proc. Natl. Acad. Sci. USA, 96, 9827 (1999); and Y. Zgao et al., Cancer Res., 61 6082 (2001)). In the present invention, the use of such a cell can minimize measurement error depending upon examiners without much more time and labor.

In the present invention, the soft agars thus constituted are dissolved on a laboratory dish at a prescribed temperature, for example, 42 degreeC to form the culturing system 1.

The culturing system 1 thus prepared is cultured under prescribed conditions. By defining the culturing conditions of the culturing system 1, the tissue culture can be carried out within a short period of time in a stable manner, giving reproducible detected and analyzed results.

For example, when culture is carried out at a temperature of about 37 degreeC in 5% carbon dioxide gas for 15 to 30 days, preferably 15 days, within an incubator, reproducible detection and analysis can be made.

(Detection System)

The detection system 2 of the detection/analysis system for caner cell colonies according to the present invention is mainly composed of a microscope 21, a digital camera 22, serving as electric data conversion means, connected to the microscope 21, and a computer system 23.

The microscope 21 used in the present invention is not specifically restricted as long as it has a conventional magnification degree, and is capable of being connected to the electric data conversion means such as the digital camera 22.

The digital camera 22 is the electric data conversion means, which converts an analog data magnified with the microscope 21 into digital image data such as JPEG, TIFF, PCX, GIF format. Dy the digital camera 22, the digital image data is stored in a storage medium possessed by the digital camera, and the stored digital image data can be taken in the computer system 23 via the storage medium. More preferably, the stored digital data is directly sent to the computer system, via an interface such as USB (Universal Serial Bus).

The computer system 23 used in the detection system 2 is mainly composed of a storage, CPU (central processing unit) for executing a program stored in the storage, RAM (Random Access

Memory), and an interface for receiving the digital image data taken by the digital camera such as USB interface, media reading means, which reads contents of a medium accommodated with the digital camera. In the storage possessed by the computer system 23, programs such as an operating system and an image analyzing software, digital image data and other data are stored.

In the present invention, the digital image data, which is results of the culture taken from the digital camera 23 is analyzed by the image analyzing software. The image analyzing software can, for example, convert the data into gray scale data, make a calibration, and subtraction, convert the data into binary data through a single threshold value, to thereby analyze it for at least one item selected from the group consisting of presence or absence of colony(ies), number of colonies; and distribution of colonies. At this time, for the purpose of acquiring a digital image through the microscope, it is preferable to make a transparency of the agar gel uniform, to treat scattered light of the microscope. More preferably, it is desired that an image differential treatment according to a conventionally known method is carried out to distinguish the colonies from dust. In this case, when the spot having a long diameter/short diameter ratio not more than 1.6 is judged to be the cancer cell colony.

When an image is taken from the microscope, at a central portion, an amount of light becomes inevitably large, and the view becomes dark as far from the center. Consequently, in

order to adjust the distribution of light amount, image differentiation is preferably carried out. As one of the methods, a laboratory disk on which an agar gel with no cell is placed is taken, and the acquired digital image data is taken as a control image. When the control image is subtracted from a sample image, which is a subject matter of the detection and analysis, a sample image whose brightness becomes uniform can be obtained.

According to such a configuration, the detection and analysis of the cells cultured in the culturing system 1 under prescribed conditions can be carried out with good efficiency and with good reproductivity.

Next, differences between the results of the detection and analysis performed with the detection/analysis system for caner cell colonies according to the present invention and those with the conventional system will be listed.

Difference Between Conventional System and Present System

Preparation of Sample, Tissue Culture

Inducing Cancer Cell Colonies (37 degreeC/5 % CO_2/14 days)

Detection/Analysis System for Measuring Method	Conventional Cancer Cell Colonies
Device for Microscopically Taking Colonies	Visibly Counting Colonies From Microscope
Results (Number, Shape, Size, Distribution)	Results (only Number of Colonies)
Evaluations	
Improvement in Accuracy/ Shortening Period/Increasing Analysis Items/ Mass Analysis with Ease/Improvement in Research Environment	Personal Difference/ Restriction of Analysis Items Mass Analysis with Difficulty Requirement of Much Works

EXAMPLES

The following Examples shows the results where using the detection/analysis system for cancer cell colonies according to the present invention, substances originated from foods having anti-cancerous effect were added to the bottom layer 11

of the culturing system 1, and then cultivation was performed to detect and analyze the cancer cell colonies.

(EXAMPLE 1)

In Example 1, the culturing system 1 was prepared by adding concentrated liquid of sweet potato juice and TPA to the agar of the bottom layer. The resulting culturing system 1 was cultured under prescribed conditions and the detection and analysis were carried out using the detection/analysis system for cancer cell colonies according to the present invention. The results are shown in FIG. 2. In the graph shown in FIG. 2, the ratio of suppressing cancerous change was taken as the vertical axis, the concentration of the sweet potato juice was taken as the horizontal axis, and the ratios of suppressing cancerous change in respective concentrations of sweet potato juice were plotted.

It can be understood from FIG. 2 that as the concentration of the sweet potato juice is increased, the ratio of suppressing cancerous change is increased.

(EXAMPLE 2)

In Example 2, the culturing system 1 was prepared by adding blueberry anthocyanin contained in blueberry to the agar of the bottom layer. The resulting culturing system 1 was cultured under prescribed conditions and the detection and analysis were carried out using the detection/analysis system for cancer cell colonies according to the present invention. The results are shown in FIG. 3. Similar to FIG. 2, in the graph shown in FIG.

3, the ratio of suppressing cancerous change was taken as the vertical axis, the concentration of the blueberry anthocyanin was taken as the horizontal axis, and the ratios of suppressing cancerous change in respective concentrations of blueberry anthocyanin were plotted.

It can be understood from FIG. 3 that as the concentration of the blueberry anthocyanin is increased, the ratio of suppressing cancerous change is increased.

(EXAMPLE 3)

In Example 3, the culturing system 1 was prepared by adding rain originated from Chinese medicine Rheum officinale (Chinese rhubarb) to the agar of the bottom layer. The resulting culturing system 1 was cultured under prescribed conditions and the detection and analysis were carried out using the detection/analysis system for cancer cell colonies according to the present invention. The results are shown in FIG. 4. Similar to FIG. 2, in the graph shown in FIG. 4, the ratio of suppressing cancerous change was taken as the vertical axis, the concentration of the rain was taken as the horizontal axis, and the ratios of suppressing cancerous change in respective concentrations of rain were plotted.

It can be understood from FIG. 3 that as the concentration of rain is increased, the ratio of suppressing cancerous change is increased.

(EXAMPLE 4)

In Example 4, the culturing system 1 was each prepared

by adding peonidin malvidin, pelargonoidin, cyanidin, and delphinidin contained in anthocyanidin respectively to the agar of the bottom layer. The resulting culturing system 1 was cultured under prescribed conditions and the detection and analysis were carried out using the detection/analysis system for cancer cell colonies according to the present invention. The results are shown in FIG. 5. Similar to FIG. 2, in the graph shown in FIG. 5, the ratio of suppressing cancerous change was taken as the vertical axis, the concentration of each component originated from anthocyanidin was taken as the horizontal axis, and the ratios of suppressing cancerous change in respective concentrations of each component were plotted.

It can be understood from FIG. 3 that as the concentration of each component is increased, the ratio of suppressing cancerous change is increased. Particularly, it can be proven that delphinidin is of highly effective for suppressing cancerous change.

As described above, as shown in Examples 1 to 4, the culturing system and the detection/analysis system according to the present invention can be used to quantitatively measure ratio for suppressing cancerous change.

INDUSTRIAL APPLICABILITY

According to the present invention, cancerous cell inducers in an environment which cancerously change normal cells, chemicals and food which suppress cancerous change of normal cells can be rapidly and surely detected, providing

effective evidence means for preventing cancer by food materials for long life and healthy society.